AVIAN DISEASES 46:219–223, 2002

Research Note—

Direct Polymerase Chain Reaction Detection of Campylobacter spp. in Poultry Hatchery Samples

Kelli L. Hiett, Nelson A. Cox, and Norman J. Stern

United States Department of Agriculture, Agricultural Research Service, Poultry Microbiological Safety Research Unit, Russell Research Center, P.O. Box 5677, Athens, GA 30604-5677

Received 27 July 2001

SUMMARY. A rapid, sensitive, and specific polymerase chain reaction (PCR) assay was developed for the direct detection of *Campylobacter* in environmental samples from hatcheries. PCR, with a set of primers specific for the *Campylobacter fla*A short variable region (SVR), detected the presence of *Campylobacter* in both fluff and eggshell samples; however, a determination of whether the organism was living or dead could not be made. Conventional cultural methods detected no *Campylobacter* from the same samples. An additional benefit of the direct PCR assay is it allows for the production of a product that can be sequenced to provide further epidemiologic information.

RESUMEN. *Nota de Investigación*—Detección directa de Campylobacter spp en muestras de plantas de incubación.

Se desarrolló un ensayo rápido, sensible y específico de reacción en cadena de la polimerasa (de las siglas en inglés PCR) para la detección directa de *Campylobacter* en muestras de medio ambiente de plantas de incubación. Mediante la prueba de PCR y utilizando un par de iniciadores específicos para *Campylobacter flaA* SVR, se detectó la presencia de *Campylobacter* en muestras de plumón y cascarones de huevo; sin embargo, no se pudo determinar si el organismo se encontraba vivo o muerto. No se detectó *Campylobacter* en las mismas muestras mediante métodos convencionales de cultivo. Una ventaja adicional de la prueba de PCR directa es que permite la obtención de un producto cuya secuencia puede ser analizada para posteriormente obtener información epidemiológica.

Keywords: *Campylobacter,* PCR, rapid detection, hatchery

Abbreviations: PCR = polymerase chain reaction; SVR = short variable region

Campylobacter jejuni, a gram-negative, microaerophilic bacteria, is presently believed to be the leading bacterial etiologic agent of acute gastroenteritis in the human population; the total number of *Campylobacter* enteritis cases in the United States is estimated at 2.4 million per year, or approximately 1%–2% of the population per year (2,26,30,31,32). Handling and consumption of poultry or poultry related products are considered to be a primary source for *Campylobacter*-induced disease in humans (3,16,21). *Campylobacter* has been cultured from as many as 75% of the live broiler population and from as much as 80% of processed poultry meat samples sold commercially (10,11,24). The high colonization incidence of poultry and the resultant clinical infections in humans have prompted a number of investigations focused upon identifying and subsequently eliminating sources of *Campylobacter* contamination in chickens.

Several suspected horizontal sources or vectors of infection include the broiler house environment, litter, feed, water, personnel, small animals on the farm, flies, and rodents (8,12,15,27). Transmission of *Campylobacter* from the breeder flock has traditionally been dismissed as a source of entry primarily because of the inability to culture *Campylobacter* from hatchery samples or from newly hatched chicks (1,7,14,25). The detection of *Campylobacter* in the aforementioned epidemiologic investiga-

tions has generally relied upon traditional cultural methodology followed by microscopy and serologic tests for confirmation. These techniques require several days to complete and often lack sensitivity (9,17,18,22,23,28,33,34, 35). Additionally, the occurrence of viable but nonculturable forms of *Campylobacter* further complicates detection.

A more rapid and sensitive technique, the polymerase chain reaction (PCR), has recently been employed for the detection of *Campylobacter*. Several PCR assays have been developed that identify *Campylobacter* from pure cultures or from products that have been artificially inoculated (9,17,18,22,33,34,35,36,37). However, many of these assays have not been extended to demonstrate detection in naturally contaminated samples, such as food products, environmental samples, and feces, where other components can interfere with the reaction. In this paper, we describe a PCR assay, with a previously developed set of primers specific for a highly conserved region of the *fla*A gene of *Campylobacter,* to test native hatchery fluff and eggshells for the presence of *Campylobacter*. This new methodology was demonstrated to be rapid, powerful, and sensitive.

MATERIALS AND METHODS

Collection of hatchery samples. Approximately 40 g of eggshells and 44 g of dry fluff were collected individually in plastic bags and transported to the laboratory.

Cultural methodology. Twenty grams of each eggshell sample and 22 g of each fluff sample were individually enriched in Bolton broth at 37 C for 4 hr followed by a 20-hr incubation at 42 C in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen). Both direct platings and serial dilutions of the enrichment broth were plated onto Campy-Cefex agar and incubated at 42 C, microaerobic conditions, for 48 hr.

Template preparation and *fla*A SVR gene amplification by PCR. Approximately 1 g of eggshell or 1 g of fluff was placed individually into sterile 50-ml centrifuge tubes. Sterile water (50 ml) was added to each of the samples; samples were vigorously shaken. Five hundred microliters of liquid was removed from each sample, placed into a sterile microcentrifuge tube, and placed at 100 C for 10 min. Fifty microliters of each boiled sample was used as template for *fla*A short variable region (SVR) PCR. Primers used in the reactions were FLA242FU: 5′CTA TGG ATG AGC AAT TWA AAA T 3′ and FLA625RU: 5′CAA GWC CTG TTC CWA CTG AAG3′. The concentration of $MgCl_2$ in the reaction was 1.5 mM. Cycling conditions in a hot start PCR were as follows: 35 cycles of 94 C 1 min, 50 C 1 min, 72 C 1 min. Ten microliters of each sample was removed from each reaction and electrophoretically resolved in a 1% Seakem LE agarose gel.

Both positive and negative controls were included with each set of PCRs performed. For the positive control, an isolated colony of *Campylobacter* was resuspended in 300 μl of sterile H_2O and placed at 100 C for 10 min. Ten microliters of the boiled cell suspension was used as a template for *fla*A SVR PCR analysis. Amplification and electrophorectic resolution were performed as described above. For the negative controls, 50 μl of boiled sterile water was utilized as the template for *fla*A SVR PCR. Again, amplification and electrophoretic resolution were performed as previously described.

SVR *fla*A DNA sequence analysis. Amplified product was sequenced with either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA). Reactions were electrophoretically resolved with an ABI 3700 automated fluorescent DNA sequencer. Data were assembled with Sequencher 4.0.1 (GeneCodes Corp., Ann Arbor, MI) and aligned with ClustalX. Aligned sequences were compared and dendograms generated with the UPGMA algorithm with absolute distance measurements in PAUP*4.0 (30).

RESULTS

In an effort to detect the presence of *Campylobacter* spp. in the hatchery environment, 10 samples each of both fluff and eggshells were collected from four individual hatching cabinets, and subjected to both traditional cultural techniques and PCR. Results are listed in Table 1. All samples, fluff (40 samples) and eggshell (40 samples), tested negative by cultural methodology. PCR analysis of fluff yielded an amplicon, approximately 400 base pairs in length, in all samples tested (40/40) (Fig. 1). This amplicon size is consistent with that expected for amplification of the SVR of the *fla*A gene of *Campylobacter* spp. PCR analysis of eggshell samples yielded product of the expected size in 70% (28/40) of all samples tested. Subsequent DNA sequence analyses of selected PCR products were consistent with the amplicon arising from the SVR of the *fla*A gene of *Campylobacter* spp. (data not shown). However, sequence data revealed that some PCR amplicons arose from the amplification of more than one distinct

Table 1. Culture and PCR data.[A]

	Hatching cabinet															
	A				B				C				D			
	Fluff		Eggshells		Fluff		Eggshells		Fluff		Eggshells		Fluff		Eggshells	
	C	P	C	P	C	P	C	P	C	P	C	P	C	P	C	P
1	−	+	−	+	−	+	−	−	−	+	−	+	−	+	−	−
2	−	+	−	+	−	+	−	−	−	+	−	−	−	+	−	+
3	−	+	−	+	−	+	−	−	−	+	−	+	−	+	−	−
4	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	−
5	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+
6	−	+	−	+	−	+	−	+	−	+	−	−	−	+	−	+
7	−	+	−	+	−	+	−	−	−	+	−	+	−	+	−	−
8	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	−
9	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
10	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+

[A]C = detection by cultural method; P = detection by PCR method; + = positive; − = negative.

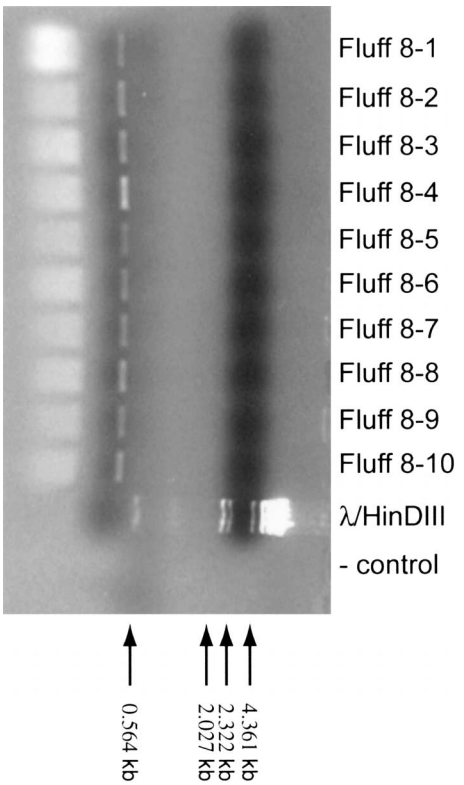

Fig. 1. PCR amplification and detection of *Campylobacter fla*A SVR. Lanes 1–10, amplicons from fluff samples taken from hatching cabinet A. Lane 11, a λ/*Hin*DIII marker. Lane 12, a negative control. The positive control is not shown on this gel.

Campylobacter clone; in other words, multiple *Campylobacter* clones were present in the original fluff and eggshell samples.

DISCUSSION

The Centers for Disease Control and Prevention estimates that *Campylobacter* enteritis is a multi-billion dollar disease and that the consumption of poultry is a primary source for resultant clinical infections in humans. An understanding of the pathways involved in *Campylobacter* contamination of poultry flocks is therefore essential for the development of intervention strategies and the subsequent reduction of *Campylobacter* in poultry. Traditionally, the detection of *Campylobacter* in epidemiologic investigations has relied upon cultural methodology followed by microscopy and serologic tests for confirmation. This method of detection requires several days to complete and often lacks sensitivity (9,17,18,22,33,34,35,36,37). The PCR is a technology that offers a sensitive, specific, and rapid alternative for the detection of *Campylobacter*. However, PCR alone has not been extended to demonstrate detection of *Campylobacter* in naturally contaminated samples, such as food products, environmental samples, or feces.

The detection of specific microorganisms by PCR is highly dependent upon the sensitivity, specificity, and robustness of the primer set used. Meinersmann *et al.* (20) developed a set of primers that flank a highly variable region,

termed the SVR, of the *Campylobacter fla*A gene. Sequence analysis of the *Campylobacter fla*A SVR was shown to be a useful subtyping tool in epidemiologic investigations. In subsequent investigations, these primers were demonstrated to be very sensitive, amplifying as few as 1.25 organisms (unpubl. data). Additionally, the *fla*A SVR primers were tested for specificity against a variety of poultry-associated microorganisms commonly detected in broiler houses. These organisms included *Staphylococcus aureus, Enterococcus faecalis, Proteus vulgaris, Enterobacter cloacae, Shigella sonnei, Salmonella enteritidis, Escherichia coli, Pseudomonas aeruginosa, Citrobacter freundii, Salmonella typhimurium, Klebsiella pneumoniae, Clostridium perfringens, Pasteurella multocida, Bordetella avium, Haemophilus paragallinarium,* and *Listeria monocytogenes* (unpubl. data). *Escherichia coli* was the only organism that produced products with the *fla*A SVR primer set; two products were observed, one approximately 1400 base pairs and one approximately 700 base pairs. The resulting *E. coli* products were readily distinguished from those of *Campylobacter* by both number and size.

The established sensitivity and specificity of the *fla*A SVR primer set prompted us to develop a direct PCR assay capable of detecting *Campylobacter* from naturally contaminated environmental samples. Hatchery samples were chosen as the test material because of the newly emerging hypothesis of breeder to broiler transmission. Traditionally, the transmission of *Campylobacter* from the breeder flock to the broiler offspring has been dismissed because of the inability of cultural methods to detect *Campylobacter* from hatchery samples or from newly hatched chicks (1,7,14,25). Recently, however, several published studies suggest circumstantially that egg transmission from one generation to the next is possible (4,5,6,13,19,22). In this study, we demonstrated that *Campylobacter* DNA is indeed present in both fluff and eggshells. However, a determination of whether the organism is living or dead could not be made. Further experiments with these primers in a reverse transcription PCR assay should address the question of viability. In conclusion, we demonstrate that *fla*A SVR PCR provides a reliable and convenient means for rapidly assessing *Campylobacter* contamination in environmental samples. Additionally, this method will allow for the production of a product that can be sequenced to provide further epidemiologic information. We expect that the described assay will also be adapted to assess *Campylobacter* contamination in other environmental samples and poultry products.

REFERENCES

1. Acuff, G. R., C. Vanderzandt, F. A. Gardner, and F. A. Golan. Examination of turkey eggs, poults, and brooder house facilities for Campylobacter jejuni. J. Food Prot. 45:1279–1281. 1982.

2. Blaser, M. J., and L. B. Reller. Campylobacter enteritis. N. Engl. J. Med. 305:1444–1452. 1981.

3. Bryan, F. L., and M. P. Doyle. Health risks and consequences of Salmonella and Campylobacter jejuni in raw poultry. J. Food Prot. 58:326–344. 1995.

4. Chuma, T., T. Yamada, K. Yano, K. Okomoto, and H. Yugi. A survey of Campylobacter jejuni in broilers from assignment to slaughter using DNA-DNA hybridization. J. Vet. Med. Sci. 56:697–700. 1994.

5. Clark, A. G., and D. H. Bueschkens. Laboratory infection of chicken eggs with Campylobacter using pressure differential. Appl. Environ. Microbiol. 49:1467–1471. 1985.

6. Cox, N. A., N. J. Stern, K. L. Hiett, and M. E. Berrang. Identification of a new source of Campylobacter contamination in poultry: transmission from breeder hens to broiler chickens. Avian Dis. In press. 2002.

7. Doyle, M. P. Association of Campylobacter with laying hens and eggs. Appl. Environ. Microbiol. 47:533–536. 1984.

8. Genigeorgis, C. A., M. Hassuney, and P. Collins. Campylobacter jejuni infection on poultry farms and its effect on poultry meat contamination during slaughtering. J. Food Prot. 49:895–903. 1986.

9. Giesendorf, B. A. J., W. G. V. Quint, M. H. C. Henkens, H. Stegemen, F. A. Huf, and H. G. Niesters. Rapid and sensitive detection of Campylobacter spp. in chicken products by using polymerase chain reaction. Appl. Environ. Microbiol. 58:3804–3808. 1992.

10. Hood, A. M., A. D. Pearson, and M. Shalamat. The extent of surface contamination of retailed chickens with Campylobacter jejuni serogroups. Epidemiol. Infect. 100:17–25. 1988.

11. Izat, A. L., and F. A. Gardner. Marketing and products: incidence of Campylobacter jejuni in processed egg products. Poult. Sci. 67:1431–1435. 1986.

12. Jacobs-Reitsma, W. F. Aspects of epidemiology of Campylobacter in poultry. Vet. Q. 19:113–117. 1997.

13. Jacobs-Reitsma, W. F. Experimental horizontal spread of Campylobacter amongst one-day-old broilers. In: Campylobacter, Helicobacter and related or-

ganisms, 1st ed. A. J. Lastovica, D. G. Newell, and E. E. Lastovica, eds. University of Cape Town, Cape Town, South Africa. pp. 377–378. 1998.

14. Jones, F. T., R. C. Axtrell, D. V. Rives, S. E. Scheidleler, F. R. Tarver, R. L. Walker, and M. J. Wineland. A survey of Campylobacter jejuni contamination in modern broiler production and processing systems. J. Food Prot. 54:259–262. 1991.

15. Kazwala, R. R., J. D. Collins, J. Hannin, A. P. Crinion, and H. O'Mahoney. Factors responsible for the introduction and spread of Campylobacter jejuni infection in commercial poultry production. Vet. Rec. 126:305–306. 1990.

16. Kinde, H., C. A. Genigeorgis, and M. Pappaioanou. Prevalence of Campylobacter jejuni in chicken wings. Appl. Environ. Microbiol. 45:1116–1118. 1983.

17. Kirk, R., and M. T. Rowe. A PCR assay for the detection of Campylobacter jejuni and Campylobacter coli in water. Lett. Appl. Microbiol. 19:301–303. 1994.

18. Lawson, A. J., D. Linton, J. Stanley, and R. J. Owen. Polymerase chain reaction detection and speciation of Campylobacter upsaliensis and C. helveticus in human faeces and comparison with culture techniques. J. Appl. Microbiol. 83:375–380. 1997.

19. Lindblom, G. B., E. Sjogren, and B. Kaijser. Natural Campylobacter colonization in chickens raised under different environmental conditions J. Hyg. (Lond.) 96:385–391. 1986.

20. Meinersmann, R. J., L. O. Helsel, P. I. Fields, and K. L. Hiett. Discrimination of Campylobacter jejuni isolates by fla gene sequencing. J. Clin. Microbiol. 33:2810–2814. 1997.

21. Park, C. E., Z. K. Stankiewicz, J. Lovett, and J. Hunt. Incidence of Campylobacter jejuni in fresh eviscerated whole market chickens. Can. J. Microbiol. 27:841–842. 1981.

22. Pearson, A. D., M. H. Greenwood, R. K. Feltham, T. D. Healing, J. Donaldson, D. M. Jones, and R. R. Colwell. Microbial ecology of Campylobacter jejuni in a United Kingdom chicken supply chain: intermittent common source, vertical transmission, and amplification by flock propagation. Appl. Environ. Microbiol. 62:4614–4620. 1996.

23. Rasmussen, H. N., J. E. Olsen, K. Jorgensen, and O. F. Rasmussen. Detection of Campylobacter jejuni and Camp. coli in chicken faecal samples by PCR. Lett. Appl. Microbiol. 23:363–366. 1996.

24. Rosef, O., B. Gondroson, and G. Kapperud. Campylobacter jejuni and Campylobacter coli as surface contaminants of fresh and frozen poultry carcasses. Int. J. Food Microbiol. 1:205–215. 1984.

25. Shanker, S., A. Lee, and T. C. Sorrell. Campylobacter jejuni in broilers: the role of vertical transmission. J. Hyg. (Lond.). 96:153–159. 1986.

26. Slutsker, L., S. F. Altekruse, and D. L. Swerdlow. Foodborne diseases. Emerging pathogens and trends. Infect. Dis. Clin. North Am. 12:199–216. 1998.

27. Smitherman, R. E., C. A. Genigeorgis, and T. B. Farver. Preliminary observations on the occurrence of Campylobacter jejuni at four California chicken ranches. J. Food Prot. 47:293–298. 1984.

28. Stonnet, V., L. Sicinschi, F. Megraud, and J. L. Guesdon. Rapid detection of Campylobacter jejuni and Campylobacter coli isolated from clinical specimens using the polymerase chain reaction. Eur. J. Clin. Microbiol. Infect. Dis. 14:335–359. 1995.

29. Swofford, D. L. PAUP*: phylogenetic analysis using parsimony and other methods, version 4.0. Sinauer Associates, Sunderland, MA. 1988.

30. Tauxe, R. V. Epidemiology of Campylobacter jejuni infections in the United States and other industrialized nations. In: Campylobacter jejuni: current status and future trends, 1st ed. I. Nachamkin, M. J. Blaser, and L. S. Thompkins, eds. American Society for Microbiology, Washington, DC. pp. 9–19. 1992.

31. Tauxe, R. V., N. Hargrett-Bean, C. M. Patton, and I. K. Wachsmuth. Campylobacter isolates in the United States, 1982–1986. MMWR CDC Surveill. Summ. 37:1–13. 1988.

32. Taylor, D. N. Campylobacter infections in developing countries. In: Campylobacter jejuni: current status and future trends, 1st ed. I. Nachamkin, M. J. Blaser, and L. S. Thompkins, eds. American Society for Microbiology, Washington, DC. pp. 20–30. 1992.

33. Thunberg, R. L., T. T. Tran, and M. O. Walderhaug. Detection of thermophilic Campylobacter spp. in blood-free enriched samples of inoculated foods by the polymerase chain reaction. J. Food Prot. 63:299–303. 2000.

34. Vanniasinkam, T., J. A. Lanser, and M. D. Barton. PCR for the detection of Campylobacter spp. in clinical specimens. Lett. Appl. Microbiol. 28:52–56. 1999.

35. Waage, A. S., T. Vardund, V. Lund, and G. Kapperud. Detection of small numbers of Campylobacter jejuni and Campylobacter coli cells in environmental water, sewage, and food samples by a seminested PCR assay. Appl. Environ. Microbiol. 65: 1636–1643. 1999.

36. Wang, H., J. M. Farber, N. Malik, and G. Sanders. Improved PCR detection of Campylobacter jejuni from chicken rinses by a simple sample preparation procedure. Int. J. Food Prot. 52:39–45. 1999.

37. Winters, D. K., A. E. O'Leary, and M. F. Slavik. Polymerase chain reaction for rapid detection of Campylobacter jejuni in artificially contaminated foods. Lett. Appl. Microbiol. 27:163–167. 1998.